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Automated Enzymatic Methods for Creatinine Measurement with Special Attention to Bilirubin Interference

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Summary: Four enzymatic methods for creatinine measurement were evaluated on a DuPont Dimension™ automatic analyzer. Biomed Creatinine-Duo-UV (BIO) and Raichem™ Creatinine Reagent Enzymatic (RAI) start creatinine breakdown with creatinine iminohydrolase (EC 3.5.4.21) resulting in the formation of NH_4^+. The Boehringer Mannheim Creatinine PAP (BM1) and SopaChem Creatinine (SOP) start the breakdown of creatinine with creatininase (EC 3.5.2.10) which yields creatine. In order to reduce bilirubin interference, the BM1 method was modified to contain $K_4Fe(CN)_6$. This substance was added with reagent 1 (BM2) or with reagent 2 (BM3). All the enzymatic creatinine methods tested displayed good linearity for concentrations up to at least 1000 μmol/l. The BIO, BM3, RAI and SOP methods showed good stability of test outcome for the tested period of a week. The outcome of the BM1 and BM2 method increased continually with time. Only the results of the RAI method were diminished by the presence of lipids. The BM1, BM2, BM3 and SOP methods showed no interference with haemoglobin, whereas this increased the outcome of the BIO method and slightly decreased the results of the RAI method. Using spiked human albumin solutions it was found that the BIO, BM2, BM3 and RAI methods displayed good resistance to interference by bilirubin or ditauro-bilirubin. The outcome of the BM1 and SOP method was strongly decreased by both bilirubin and ditauro-bilirubin. When creatinine was measured in a panel of sera originating from 100 patients with bilirubin concentrations higher than 50 μmol/l, the obtained results were in close agreement with those found for the spiked human albumin solutions. Considering the conditions evaluated, our conclusion is that the Boehringer Mannheim Creatinine PAP method adapted to contain $K_4Fe(CN)_6$ in reagent 2 (BM3) performs best of the methods tested on the DuPont Dimension™ automatic analyzer.

Introduction

Creatinine is formed from creatine and creatine phosphate at a rate directly related to total muscle mass and, thus, roughly to body weight. Because creatinine is produced at a fairly constant rate and is not reused in the body, the serum concentration is almost completely dependent on the creatinine clearance. As creatinine is cleared exclusively by glomerular filtration, serum creatinine concentration is accepted in clinical medicine as the most indicative measure of renal function (1, 2).

The classical method for the measurement of creatinine is based on the reaction with picrate under alkaline conditions as described in 1886 by *Jaffé* (3). Ever since this method was first used to quantify creatinine (4), it has been considered unsatisfactory and much effort has gone into circumventing the limitations of this technique (1, 5, 6). Nowadays the *Jaffé* creatinine test is usually configured to a two-point, fixed time, kinetic measurement with or without correction algorithm (7). In this way the fast reaction rate of creatinine compared to that of interfering chromogens is employed to improve test performance (1).

Methods employing enzymatic breakdown of creatinine are an alternative to the *Jaffé*-type creatinine

assay. These enzyme-based methods can follow two principally different reaction schemes. In the first reaction scheme creatinine is deaminated by creatinine deaminase (creatinine iminohydrolase, EC 3.5.4.21) yielding NH_4^+. This compound is measured in a subsequent reaction consuming NADPH or NADH which can be monitored photometrically. In the second reaction scheme creatinine is hydrolysed to creatine by creatininase (EC 3.5.2.10). The following breakdown of the formed creatine results eventually in the formation of H_2O_2, which is used for the formation of a quinone-imine dye by peroxidase.

In this study we evaluated four commercial enzymatic creatinine assays using a DuPont dimension™ automatic analyzer. Two methods are based on the creatinine iminohydrolase route: Biomed Creatinine-Duo-UV (BIO) and Raichem™ Creatinine Reagent Enzymatic (RAI). The other two methods are based on the creatininase route: Boehringer Mannheim Creatinine PAP (BM1) and SopaChem Creatinine (SOP).

A major problem in creatinine measurement is the interference caused by bilirubin. This is especially true for *Jaffé* methods (1). However, the Boehringer Mannheim Creatinine PAP method also proved to be prone to bilirubin interference (8). Adaptation of this method by addition of $K_4Fe(CN)_6$, which increases the specificity of the colour reaction (9), resulted in better performance (10). The original Boehringer Mannheim Creatinine PAP method is evaluated together with two modifications: $K_4Fe(CN)_6$ was added with the first (BM2) or with the second reagent (BM3).

Interference caused by bilirubin was investigated in two ways. Firstly, solutions of creatinine in human albumin spiked with unconjugated or conjugated (ditauro-) bilirubin were assayed. Secondly, icteric patient specimens were analyzed and compared to the HPLC reference method (11).

Materials and Methods

Materials

Bilirubin, creatinine and potassium ferrocyanide ($K_4Fe(CN)_6$) were obtained from Merck (Darmstadt, Germany); conjugated bilirubin (sodium salt of ditauro-bilirubin) from Porphyrin Products Inc. (Logan, UT, USA); human albumin from Behring Werke (Marburg, Germany); Intralipid™ (a 20% fat emulsion for intravenous injection) from Kabi Pharmacia b. v. (Woerden, The Netherlands); TestPoint unassayed chemistry control serum level 1 and 2 from Technicon (Tarrytown, NY, USA); Biomed Creatinine-Duo-UV from MP Products (Soest, The Netherlands); Boehringer Mannheim Creatinine PAP from Boehringer Mannheim (Mannheim, Germany); SopaChem Creatinine from Sopar-Biochem n. v. (Nieuwegen, The Netherlands); and Raichem™ Creatinine Reagent Enzymatic from Reagent Applications, Inc. (San Diego, CA, USA). All water used for this study was purified by a MilliQ system (Millipore, Bedford, MA, USA).

Modifications of enzymatic creatinine tests

As the maximal test duration on the DuPont Dimension™ is limited to 12 minutes and the BIO method required a preincubation of 3 minutes, we had to shorten its incubation time to 9 instead of the recommended 20 minutes. For reasons of compensation we tripled the amount of starting reagent (reagent 2). For the RAI method 1 ml reagent 2 (starter reagent) was diluted with 2 ml of MilliQ water. This was done because the test manual prescribed addition of 7 µl reagent 2 and the Dimension™ automatic analyzer for reasons of accuracy needed to add a minimum volume of 20 µl. For the BM2 method 8 mg of $K_4Fe(CN)_6$ was dissolved in 20 ml of reagent 1. For the BM3 method 3 mg $K_4Fe(CN)_6$ was added to 5 ml of reagent 2 (starter reagent). For both BM2 and BM3 the amount of $K_4Fe(CN)_6$ represents a fair excess as this substance functions as a catalyst and is regenerated during the final colour-forming reaction.

Preparation of spiked albumin solutions for interference studies

Starting from stock solutions of 47 g/l human albumin in 50 mmol/l Tris/HCl pH 7.4, 6 mmol/l creatinine in 20 mmol/l HCl and 10 mmol/l bilirubin or ditauro-bilirubin in 100 mmol/l NaOH, solutions were made containing 40 g/l albumin, 100 or 300 µmol/l creatinine and a bilirubin or ditauro-bilirubin concentration between 0 and 1000 µmol/l (7). These solutions were prepared and kept shielded from light. Using a solution of haemolyzed erythrocytes in MilliQ water or Intralipid™, similar solutions were made containing variable concentrations of, respectively, haemoglobin or lipids. All solutions were stored at −70 °C until further use.

Patient samples

Serum specimens used in this study were collected in various departments in the Academic Hospital Rotterdam. All samples were stored at −70 °C until use. Creatinine concentration was verified by the HPLC reference method (11). Total bilirubin concentrations was measured using a routine DuPont bilirubin Flex™ for the Dimension™ analyzer. The distribution of bilirubin concentrations in the patient sera used to evaluate bilirubin interference is given in table 1.

Tab. 1. Frequency distribution of creatinine and total bilirubin concentrations in the panel of patient sera used for evaluation of bilirubin interference. The creatinine and bilirubin concentrations mentioned are not interrelated.

Concentration range (µmol/l)	Number of samples	
	Creatinine	Bilirubin
< 50	18	–
50 – 100	52	6
101 – 150	8	20
151 – 200	4	24
201 – 250	6	12
251 – 300	3	7
301 – 350	–	5
351 – 400	–	6
401 – 450	2	5
451 – 500	2	3
501 – 550	–	4
551 – 600	1	4
> 600	4	4

Instrumentation

The enzymatic methods for creatinine measurement were evaluated on a DuPont Dimension™ ES Clinical Chemistry System automatic analyzer. The analyzer used software version ES 1.1.

Statistical analysis

Regression analysis was done using the orthogonal *Deming* procedure. Intra-assay variation was calculated using series of 30 repetitive measurements of TestPoint unassayed chemistry control serum level 1 containing 93 µmol/l creatinine. Inter-assay variation was determined using results of this same control serum measured in 12 assays performed on different days.

Results

Assay conditions provided with the reagents were transformed for use on the DuPont Dimension™ analyzer. As kinetic measurement yielded worse results in terms of precision than end-point measurement, the latter was the method of choice. For the methods employing the enzyme creatinine iminohydrolase (BIO and RAI) it was necessary to optimize the conditions. For an optimal results of the BIO method it was essential to restrict the mixing force to 'gentle', as stronger mixing led to an unacceptably high variation in the results. This probably resulted from the formation of foam interfering with correct measurement of absorbance in the case of non-delicate mixing. Assay conditions of the different methods are listed in table 2. The within-run and day-to-day imprecision found are given in table 3.

The enzymatic creatinine methods were tested for linearity using aqueous standards. For the BIO and RAI method the ratio between reagent 1 and sample was set to yield a linear result up to 1000 µmol/l creatinine. BMx (BM1, BM2 and BM3) and SOP methods yielded linear results for creatinine concentrations up to 2000 µmol/l. Linearity of the enzymatic methods was also investigated using a panel of 45 serum samples with varying creatinine concentrations up to 1000 µmol/l. These patient sera were not icteric, haemolytic or lipaemic. The results of these investigations are summarized in table 4 and closely agree with the results obtained with aqueous creatinine standards.

Stability of the reagents was verified using two control sera with creatinine concentrations of 93 and 650 µmol/l and the standard provided with the SopaChem Creatinine reagent containing 177 µmol/l creatinine. The methods were calibrated once and, subsequently, fresh samples were repeatedly analyzed, using the same reagents during a period of seven days. Figure 1 shows the results that were obtained using the TestPoint level 1 control serum with 93 µmol/l cre-

Tab. 2. Assay conditions of enzymatic methods for creatinine measurement

	BIO	BMx**	RAI	SOP
Sample				
time of addition (s)	0	0	0	0
volume (µl)	50	10	50	15
wash (µl)	20	10	20	10
mix	gentle	moderate	gentle	moderate
Reagent 1				
time of addition (s)	−110	−110	−110	−110
volume (µl)	350	350	350	350
wash (µl)	0	20	0	20
mix	none	moderate	none	moderate
Reagent 2				
time of addition (s)	180	330	300	300
volume (µl)	40	70	20	70
wash (µl)	20	20	20	20
mix	gentle	moderate	gentle	moderate
Photometer				
wavelength (nm)	340	510	340	540
reference wavelength (nm)	405	700	700	700
time of measurement 1 (s)	165	300	270	270
weightfactor1*	0.875	−0.813	0.913	−0.814
time of measurement 2 (s)	720	720	720	720
weightfactor2*	−1000	1000	−1000	1000
	−1.000	1.000	−1.000	1.000

* Final result = photometry 1 × weightfactor 1 + photometry 2 × weightfactor 2
** BMx = BM1, BM2 and BM3

Tab. 3. Imprecision of enzymatic methods for creatinine measurement

	Within-run imprecision CV (%)	Day-to-day imprecision CV (%)
BIO	7.0	8.9
BM1	1.1	1.7
BM2	2.7	8.1
BM3	0.5	2.1
RAI	0.7	2.8
SOP	0.7	3.5

CV = coefficient of variation

Tab. 4. Statistical data of linearity study using non-icteric sera

	[test] = a x [HPLC] + b			
	a	b	r	$S_{y/x}$
BIO	0.912	72.5	0.986	29.5
BM1	1.050	2.0	0.998	10.5
BM2	0.998	13.8	0.998	11.9
BM3	1.046	7.6	0.998	10.5
RAI	1.038	−2.2	0.997	15.1
SOP	0.954	38.3	0.993	21.5

Method: orthogonal *Deming* procedure; range: 0 – 1000 µmol/l; n = 45

atinine. Similar results were obtained with the other two materials (results not shown). The BIO, RAI and SOP methods did not show significant change in test outcome after a week. The BM1 method showed results that increased constantly with time. This increase was boosted by the addition of $K_4Fe(CN)_6$ to reagent 1 (BM2). Surprisingly, addition of $K_4Fe(CN)_6$ to reagent 2 (BM3) resulted in a stable test outcome.

Solutions of 40 g/l human albumin containing 100 or 300 µmol/l creatinine were spiked with lipids (Intralipid™), haemoglobin, bilirubin or ditauro-bilirubin in order to investigate any interference caused by these substances. The solutions were measured using the different enzymatic methods for creatinine measurement. Figure 2 displays the results obtained using the albumin solutions with 100 µmol/l creatinine. Closely agreeing results were obtained with the solutions with 300 µmol/l (results not shown). The result of the RAI method was diminished by the presence of lipids, whereas the other methods were unaffected. Haemoglobin increased the outcome of the BIO method, whereas this slightly decreased the result of the RAI method. The BMx and SOP methods showed no interference with haemoglobin.

The BIO method was left almost unaffected by bilirubin concentrations up to 1000 µmol/l for creatinine concentrations of 100 and 300 µmol/l. Ditauro-bilirubin caused an increase in test outcome that became quite dramatic when the ditauro-bilirubin concentration was higher than 700 µmol/l. The outcome of the BM1 method was strongly diminished by both bilirubin and ditauro-bilirubin. Interference caused by these substances was reduced by addition of $K_4Fe(CN)_6$. Introduction of $K_4Fe(CN)_6$ to the sample with the first reagent (BM2) yielded a slightly better reduction of interference than introduction with the second reagent (BM3). The RAI method displayed excellent resistance to interference by both bilirubin and ditauro-bilirubin at both the creatinine concentrations investigated. The outcome of the SOP method showed only a little less interference than the unmodified Boehringer Mannheim Creatinine PAP method (BM1).

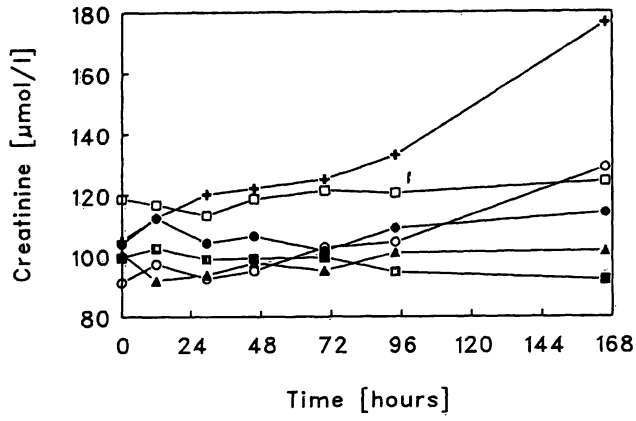

Fig. 1. Stability of results of enzymatic methods for creatinine measurement. The methods were calibrated once and subsequently fresh TestPoint unassayed chemistry control serum level 1 was analyzed for a period of a week. ● = BIO; ○ = BM1; + = BM2; ■ = BM3; ▲ = RAI; □ = SOP

Creatinine was measured in a panel of 100 sera originating from patients with total bilirubin concentrations higher than 50 µmol/l. The obtained residuals, defined as test creatinine concentration minus concentration obtained by HPLC, are shown in relation to total bilirubin concentration in figure 3. For the majority of the samples the BIO, BM2, BM3 and RAI methods yielded results that were not influenced or only slightly so by the bilirubin concentration, although especially the BIO and RAI methods show some scatter for the remaining samples. However, when residuals were plotted in relation to creatinine concentration it was found that for the BIO method imprecision was high and the test outcome was elevated in the normal and low range (results not shown). The BM1 and SOP methods were strongly affected by bilirubin. These results are in close agreement with the interference studies using bilirubin-spiked human albumin solutions.

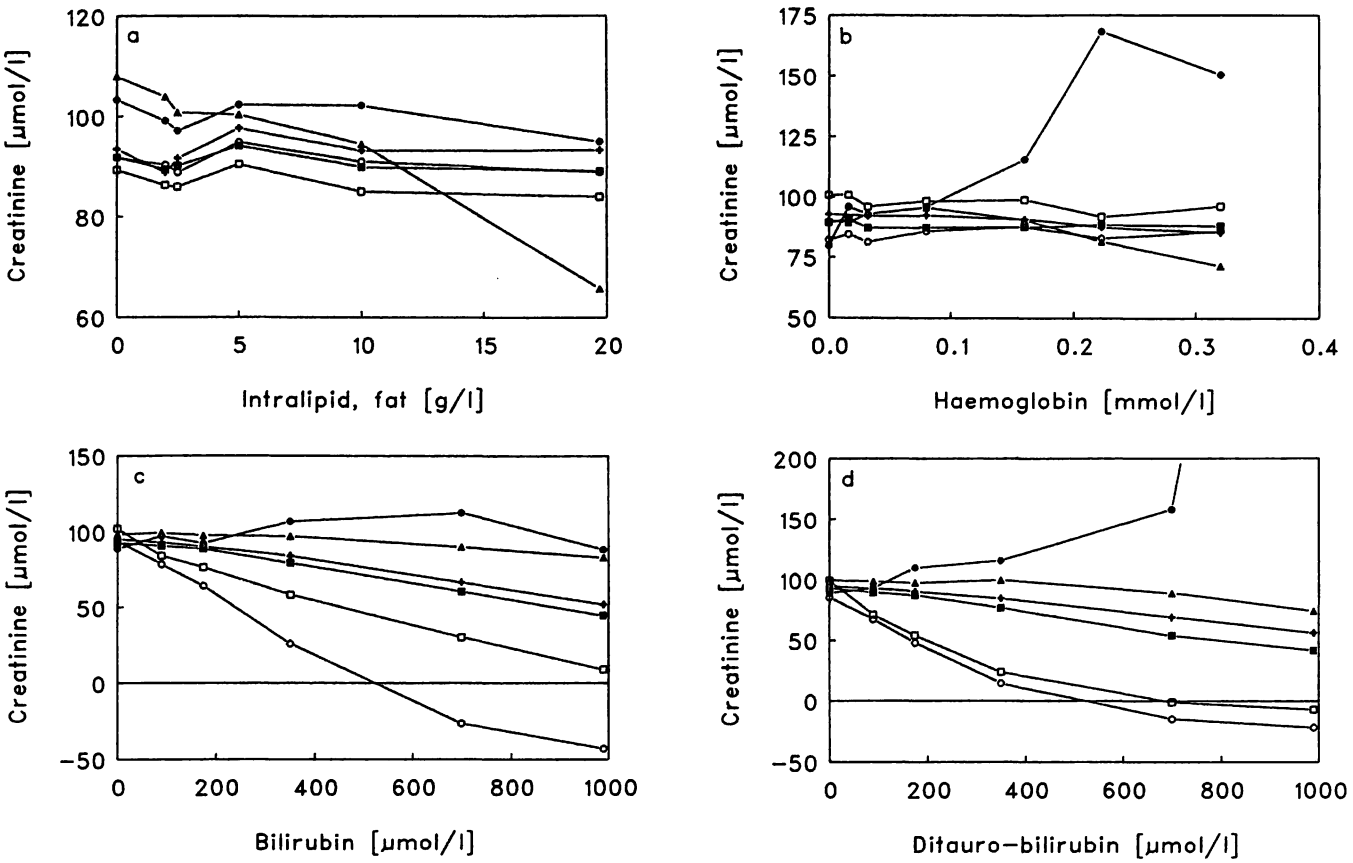

Fig. 2. Interference of enzymatic methods for creatinine measurement. Solutions of human albumin containing 100 µmol/l creatinine were spiked with possibly interfering substances.
A: interference by lipids (Intralipid™); B: interference by haemoglobin; C: interference by bilirubin; D: interference by ditauro-bilirubin
● = BIO; ○ = BM1; + = BM2; ■ = BM3; ▲ = RAI; □ = SOP

Discussion

In this study the evaluation of six automated methods for the enzymatic measurement of creatinine in serum is described. Four commercial kits were used for this purpose. A number of practical aspects regarding the performance of the various adaptations is given in the tables and the figures. Below we will try to find an explanation for some peculiarities.

For the BIO and RAI method the ratio between reagent 1 and sample was set to yield a linear result for creatinine concentrations up to 1000 µmol/l. As the set dynamic range of these tests correlates inversely with the signal amplitude at physiological creatinine concentrations and, thus, to precision at this level, the ratio is a compromise between these quantities. However, the use of a larger sample volume relatively to the BMx or SOP methods did not reduce imprecision to a level close to that displayed by the latter methods (see tab. 3).

The outcome of the BIO method was raised by haemoglobin. It has been suggested that the creatinine concentration of haemoglobin-containing solutions is raised because of endogenous creatinine from erythrocytes that is liberated upon haemolysis (12). The average creatinine concentration in erythrocytes is about 160 µmol/l (13). Because the maximal haemoglobin concentration investigated corresponds roughly to a 2% (vol/vol) erythrocyte homogenate, this phenomenon can maximally account for an increase in creatinine of about 3 µmol/l. This was confirmed by HPLC measurement of creatinine in these haemolytic solutions. Therefore, the increase in the BIO method caused by haemoglobin represents interference.

Although both the BIO and RAI methods start creatinine breakdown with creatinine iminohydrolase (EC 3.5.4.21), they display quite different degrees of variation (see tab. 3). This might be related to the use of different coenzymes, since the RAI method employs NADPH whereas the BIO method uses NADH. Use of the coenzyme NADPH instead of NADH avoids the lengthy pre-incubation otherwise required for equilibrating interfering endogenous dehydrogenase reactions (14). Therefore, the high variation in test outcome of the BIO method might be related to incomplete elimination of endogenous NH_4^+ during the pre-incubation of 180 seconds. This was supported

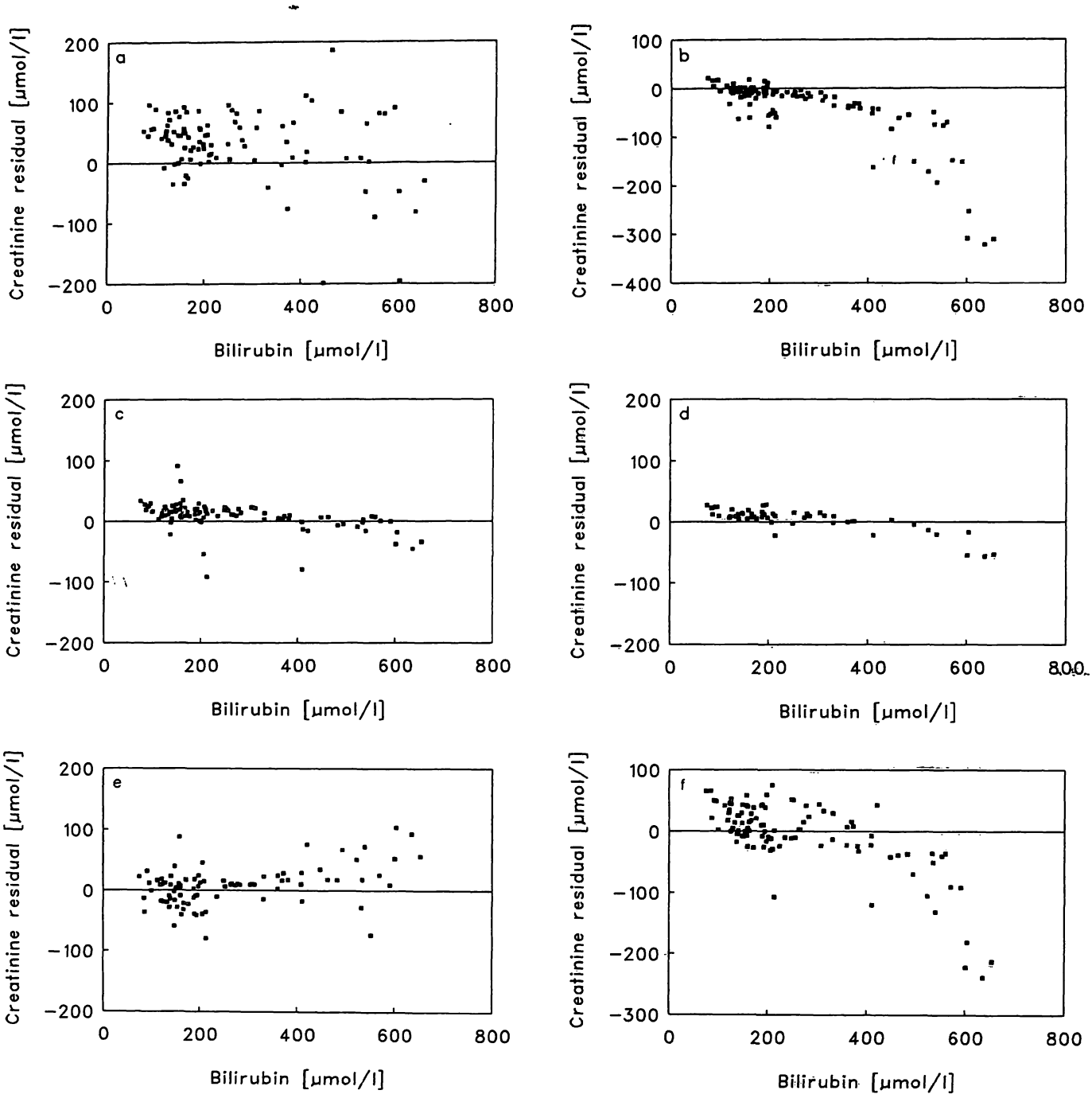

Fig. 3. Residual creatinine values of icteric sera plotted against the total bilirubin concentration. Residuals are defined as test creatinine concentration minus concentration obtained by HPLC.
a: residuals of BIO method;
b: residuals of BM1 method;
c: residuals of BM2 method;
d: residuals of BM3 method;
e: residuals of RAI method;
f: residuals of SOP method

by our findings during the optimization procedure. However, selecting end-point instead of kinetic measurement resulted in a more pronounced reduction in variation (results not shown). As the maximal test duration of the DuPont Dimension™ is limited to 12 minutes, it was not possible to implement a configuration containing both a long pre-incubation and end-point measurement.

Our findings for the BIO method implemented on the DuPont Dimension™ regarding the high variation of test outcome and the elevated results with samples containing low creatinine concentrations, are contradicted by other studies in which these problems were not encountered (15, 16). Although *Sonntag & Schumann* (15) report of rare samples with low creatinine concentrations which yielded elevated results, their

Tab. 5. Test profiles of enzymatic methods for creatinine measurement

	BIO	BM1	BM2	BM3	RAI	SOP
Linearity up to [µmol/l]	1000	2000	2000	2000	1000	2000
Stability	=	+	++	=	=	=
Variation*	+++	=	=	=	+	=
Interference						
lipids	=	=	=	=	−−	=
haemoglobin	+	=	=	=	−=	=
bilirubin	=	−−−	=	=	=	−−−
ditauro bilirubin	+=	−−−	=	=	=	−−−
Correlation with HPLC (non-icteric)	++	=	=	=	=	=
Correlation with HPLC (icteric)	++	−−−	=	=	=	−−−

+: increase of test outcome
=: constant test outcome
−: decrease of test outcome
*+: high variation
=: low variation

conclusion is that the BIO method is suited for routine creatinine measurement. This difference in outcome might result from the difference in average creatinine concentration of the samples investigated, which was lower in the present study as compared with the others (see tab. 1). The implementation of the enzymatic creatinine methods on the DuPont Dimension™ was a compromise between the recommendations of the various creatinine kit manufacturers and the capabilities of the Dimension™ analyzer. As suggested above it may be possible that lower variation can be obtained for the BIO method when using a test-configuration that runs beyond the maximal test duration of the Dimension™ analyzer.

Using the obtained results, test profiles can be compiled for the different methods (tab. 5) which contain linearity, stability, variation, interference by various substances and correlation with the HPLC reference method. As economics can nowadays not be excluded when selecting reagents, it is worth considering that the relative costs per enzymatic test are 10 to 20 times as high as for the conventional DuPont *Jaffé* creatinine Flex™ test. Taking all analytical and clinical aspects into account, together with the high price of the enzymatic creatinine methods as compared with the *Jaffé* reaction-based tests, the question arises whether these enzymatic methods are satisfactory. It is up to the reader to decide. From this study we conclude that amongst the tested enzymatic methods the Boehringer Mannheim Creatinine PAP method, adapted to contain potassium hexacyanoferrate(II) ($K_4Fe(CN)_6$) in reagent 2 (BM3), shows the best performance. Further studies are needed to determine whether the SopaChem Creatinine method, which is based on the same reaction principle, will show a comparable performance when adapted in a similar manner.

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